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Specific interaction of the new fluorescent dye 10-*N*-nonyl acridine orange with inner mitochondrial membrane

A lipid-mediated inhibition of oxidative phosphorylation

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The fluorescent dye 10-*N*-nonyl acridine orange (NAO), known as specifically associated with mitochondria, has been reported to have a cytotoxic effect when high doses were applied to cells. Presently, the biochemical basis of its toxicity was investigated on isolated rat liver mitochondria. At low concentrations, NAO strongly inhibited state 3 respiration and ATP synthesis. At high concentrations, electron transport, ATP hydrolysis, P_i-transport and adenine nucleotide activities were also decreased. All these inhibitions can be explained by probe–cardiolipin interactions which could induce the collapse of energy conversion and/or the modification of membrane fluidity.

Nonyl acridine orange; Respiratory chain, mitochondria; ATP synthesis; (Rat liver mitochondria)

1. INTRODUCTION

The fluorescent dye 10-*N*-nonyl acridine orange (NAO) was shown to bind to mitochondria of various cells [1,2] and provides a potent and new mean for the study of these organelles. Recently, we have demonstrated that the dye was accumulated into mitochondrial inner membrane independently of transmembrane potential [3]. Such a molecule, which indicates a mitochondrial membrane surface, is of interest in the analysis of membrane surface evolution during mitochondrial biogenesis [4] or during cell ageing [5]. In some fields in which mitochondrion division must be followed during several cell cycles, it is necessary to ascertain that probe–inner membrane interaction is without repercussions on cell structure and metabolism. In view of these applications, we have carried out a study on the consequence of NAO uptake by mitochondria by investigating its effect on energy-transducing activities.

2. MATERIALS AND METHODS

2.1. *Mitochondria*

Rat liver mitochondria were prepared by differential centrifugation of liver homogenate in 0.25 M sucrose, 2 mM Tris-HCl buffer (pH 7.4) as previously described [6]. For enzymatic assays of respiratory complexes, mitochondrial suspension was first stored at −20°C.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; F_1, the ATPase component of ATP synthase; F_0, the proton conducting component (membrane sector) of ATP synthase; nAt. O, nAtoms O; P_i, inorganic phosphate

2.2. *Enzymatic assays*

Rates of oxygen consumption were determined at 25°C in a medium containing 110 mM KCl, 6 mM $MgCl_2$ and 16 mM phosphate buffer (pH 7.4) under constant agitation, using a Clark oxygen electrode calibrated with sodium dithionite as described by Carafoli and Pietrobon [7].

Spectrophotometric assays of respiratory chain activities were measured on a Lambda 2 (Perkin Elmer) double-beam spectrophotometer.

Complexes **I–III** and **II–III** were assessed in 1 ml of a medium containing 50 mM Tris-HCl (pH 7.4), 50 mM NaN_3, mitochondria (25 μg protein/ml) and either 141 μM NADH or 10 mM succinate plus 0.8 μM rotenone by measuring, at 25°C, the reduction of cytochrome *c* at 550 nm. The reactions were initiated by adding 56 μM cytochrome *c* (oxidized form) [8,9].

Cytochrome *c* oxidase activity was measured by following ferrocytochrome *c* oxidation at 550 nm in 1 ml of a medium containing mitochondria (25 μg protein/ml), 10 mM phosphate buffer (pH 7.4) and 50 μM ferrocytochrome *c* [10]. The concentration of cytochrome *c* was determined using a molar extinction coefficient at 550 nm of 19 600 $M^{-1} \times cm^{-1}$ for the reduced form.

2.3. *F_0-F_1 ATPase activity*

The F_0-F_1 ATPase was assessed on mitochondria (50 μg protein/ml) by the spectrophotometric determination of NADH consumption at 340 nm using the pyruvate kinase-phosphoenolpyruvate regenerating system coupled to lactate dehydrogenase [11].

2.4. *ATP synthesis*

Intact mitochondria (1 mg protein/ml) were preincubated for 1 min at 25°C in the presence of 10 mM succinate, 0.8 μM rotenone, 225 mM sucrose, 10 mM Tris, 5 mM $MgCl_2$, 10 mM KCl, 1 mM phosphate buffer and various amounts of NAO in a final volume of 5 ml at pH 7.4. The reaction was started by adding 1 mM ADP and was stopped 5 min later by adding 0.5 ml of 40% trichloracetic acid. P_i was assayed colorimetrically [12] and net ATP synthesis was calculated from the difference in P_i present in each sample relative to a paired oligomycin (2 μg/ml) blank.

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2.5. *Adenine nucleotide translocase activity*

Translocase activity was measured at 4°C by forward exchange method [13]. Mitochondria (1 mg/ml) were briefly preincubated in 1 ml of a medium containing 16 mM phosphate buffer (pH 7.4), 6 mM $MgCl_2$, 110 mM KCl, 10 mM succinate, rotenone (1 μg/mg protein), oligomycin (5 μg/mg protein). The reaction was started by adding 50 μM [^{14}C] ADP (spec. act. 3×10^3 cpm/nmol) and stopped by addition of 20 μM carboxyatractyloside. Translocase activity was calculated as nanomoles of ADP accumulated per min per mg of protein from the radioactivity retained by mitochondria after subtracting values obtained for nonspecific retention (i.e. ADP retention by mitochondria preincubated with carboxyatractyloside before ADP addition).

2.6. *P_i transport*

Mitochondria (1 mg/ml) were incubated at 4°C in 1 ml of a medium containing 225 mM sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), 10 mM succinate and rotenone (1 μg/mg protein). The reaction was started by adding 1 mM [^{32}P]P_i (0.3 μCi) and stopped by addition of 2 mM mersalyl. P_i-transport was calculated as nanomoles of P_i transported per min per mg of protein from the radioactivity retained by mitochondria after subtracting values obtained for nonspecific retention [14].

2.7. *Protein determination*

Protein was measured by a biuret procedure using crystalline bovine serum albumin as standard [15].

2.8. *Chemicals*

10-*N*-nonyl acridine orange was a gift of H.W. Zimmerman (Institüt für Physikalische Chemie der Universität, Freiburg, FRG). ADP, CCCP and oligomycin were purchased from Boehringer; carboxyatractyloside and mersalyl from Sigma. All other reagenst were of the highest purity commercially available.

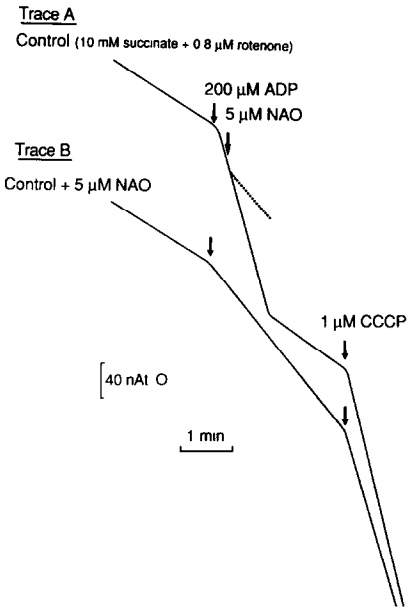

Fig.1. Polarographic traces of oxygen consumption inhibition by NAO. Intact mitochondria (1 mg/ml) were incubated at 25°C in the presence of 10 mM succinate, 0.8 μM rotenone, 16 mM phosphate buffer (pH 7.4), 6 mM $MgCl_2$, 110 mM KCl and 5 μM NAO in a final volume of 5 ml. ADP and CCCP were added as indicated. Oxygen consumption was measured polarographically with an oxygen monitor (Transidyne, General Corporation). Trace A: Initial control trace without NAO. Trace B: Initial control trace with 5 μM NAO.

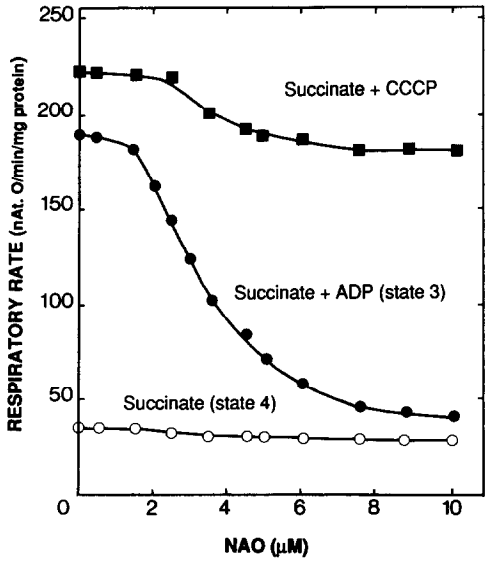

Fig.2. Oxygen consumption inhibition by NAO in the presence of FAD-linked substrate. Oxygen consumption by rat-liver mitochondria oxidizing 10 mM succinate was measured, as for fig. 1, after no additions (state 4), after 200 μM ADP (state 3) and after 1 μM CCCP (uncoupled respiration) in presence of various NAO concentrations.

3. RESULTS

3.1. *Inhibition of oxygen consumption*

A typical response to the addition of NAO to the mitochondrial suspension in the oxygen electrode chamber is shown in fig. 1. The addition of 200 μM ADP to mitochondria respiring with succinate as substrate increased the rate of oxygen consumption from 35 to 180 nAt.O/min/mg of protein (trace A). When 5 μM NAO was present, state 4 respiration remained unchanged while state 3 respiration was inhibited to 75 nAt.O/min/mg of protein (trace B).

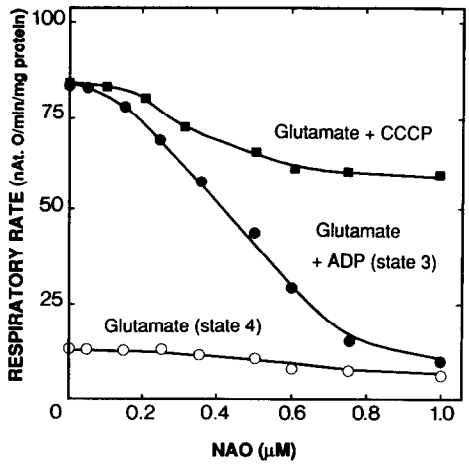

Fig.3. Oxygen consumption inhibition by NAO in the presence of NAD-linked substrate. Oxygen consumption by rat-liver mitochondria oxidizing 10 mM glutamate was measured, as for fig. 1, after no additions (state 4), after 200 μM ADP (state 3) and after 1 μM CCCP (uncoupled respiration) in presence of various NAO concentrations.

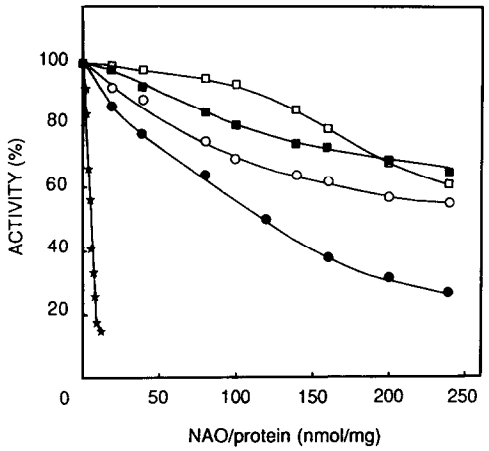

Fig.4. Effect of NAO on respiratory complexes activities, ATP hydrolysis and ATP synthesis. NADH-cytochrome *c* reductase (○); succinate-cytochrome *c* reductase (■) or cytochrome *c* oxidase (□) and F_0-F_1 ATPase (●) and ATP synthesis (★), expressed as % of control, were measured as described in section 2.

Whatever the addition order of NAO and ADP to mitochondria, the depression was almost obtained (trace A). Addition of the uncoupler CCCP to the suspension led to an oxygen consumption rate comparable to that of the control.

ADP-stimulated respiration was much more sensitive to inhibition by NAO than state 4 and uncoupled respirations (fig. 2). At 10 μM NAO, state 3 respiration was inhibited of about 75% of its control value (fig.2). Similar resuls are usually interpreted to indicate that the tested compound affects some reaction unique to the phosphorylatin pathway rather than electron transport. However, because uncoupled respiration was inhibited at relatively high NAO concentrations, at least some of the inhibition of ADP-stimulated respiration may be due to an inhibition of electron transport. Therefore, the inhibition of ADP-stimulated respiration by NAO occurred by two different mechanisms depending upon dye concentration: (i) inhibition of phosphorylation at lower concentrations (up to 3 μM) and (ii) inhibition of both phosphorylation and electron transport at higher concentrations (3–10 μM).

Similar results were obtained from rat liver mitochondria oxidizing glutamate except that the inhibition of glutamate-driven state 3 was more pronounced (fig.3). Since a given probe concentration was about 10 fold more effective on mitochondria oxidizing glutamate instead of succinate (figs 2,3), this suggests that NAO would preferentially act on the site I (complex I) region of the respiratory chain.

3.2. *Inhibition of respiratory complexes and ATPase activities*

NADH-cytochrome *c* reductase (complex **I–III**), succinate-cytochrome *c* reductase (complex **II–III**) and cytochrome *c* oxidase (complex **IV**) were less inhibited by NAO since a decrease in their activities was not greater than 50% even when NAO/protein ratio reaches the value of 240 nmol/mg protein (fig.4).

Adenosine triphosphatase was more inhibited by NAO than respiratory complexes since and inhibition of 76% was reached for the same NAO/protein ratio (240). However, much higher ratios of dye were required to inhibit ATP hydrolysis (fig.4) than were needed to inhibit ADP-stimulated respiration (fig.2).

On the contrary, NAO strongly inhibited ATP synthesis in well-coupled energized mitochondria (fig.4). At the highest NAO concentration used (10 μM), corresponding to a dye/protein ratio of 10 nmol/mg protein, ATP synthesis was inhibited by nearly 85% indicating that this step is very sensitive to probe inhibition.

3.3. *Adenine nucleotide translocase and P_i-transport activities*

At the same concentrations as those used to measure the effect of NAO on state 3 respiration and ATP synthesis, adenine nucleotide translocase activity was relatively affected since the rate of ADP transport was

Table 1

NAO effect on adenine nucleotide translocase and P_i-transport activities

Conditions	Adenine nucleotide translocase (nmol/min/mg prot.) (%)*		P_i-transport (nmol/min/mg prot.) (%)*	
Control	12.7	100	80.8	100
NAO added (μM)				
1	11.8	93	80.0	99
2	11.5	90	76.6	95
4	10.7	84	75.1	93
6	9.4	74	73.7	91
8	7.8	61	72.7	90
10	7.0	55	66.9	83

Translocase activity was measured by forward exchange method and P_i-transport into mitochondria was determined using [^{32}P]P_i as described in section 2. The translocase and P_i-transport were respectively measured 30 s and 15 s following the starting time of reaction. In the experimental conditions selected, reaction kinetics were linear up to 1.5 min for adenine nucleotide translocase and 30 s for P_i-transport. Each value represents the mean of three separate experiments.

* Adenosine nucleotide translocase and P_i-transport activities are expressed as percentage of control.

Table 2

Uptake of NAO by rat-liver mitochondria under coupled and uncoupled states

NAO added	NAO bound to mitochondria (nmol/mg protein)	
(μM)	Coupled	Uncoupled
5	4.90 ± 0.03	4.30 ± 0.11
7.5	6.80 ± 0.10	5.75 ± 0.12
10	8.75 ± 0.09	7.75 ± 0.10

Mitochondria (1 mg/ml) were incubated for 1.5 min in basic reaction medium containing 150 mM sucrose/5 mM $MgCl_2$/10 mM disodium succinate/2.5 μM rotenone/5 mM potassium Hepes buffer (pH 7.4) and various concentrations of NAO. After centrifugation for 2 min at 10 000 × *g*, free dye in the supernatant was determined by absorbance at 496 nm. NAO bound was taken as total minus free. Under uncoupled state 2 μM CCCP was added.

inhibited only to 55% of the control value in the presence of 10 μM NAO (table 1). However, P_i-trasport was only slightly inhibited since more than 80% of its original activity was maintained even when dye concentration was 10 μM (table 1).

Despite these inhibitions, these results do no entirely explain the high NAO inhibitory effects on state 3 respiration and ATP synthesis activity. Thus a possible direct action of the dye on ATP synthase complex, on its lipidic environment or on energy conservation could be at the origin of NAO effects on these functions.

3.4. *Binding of NAO to coupled and uncoupled mitochondria*

Under both coupled and uncoupled conditions the amount of NAO taken up by mitochondria increased with dye concentration in the medium (table 2). At the same range of NAO concentrations used in oxygen consumption measurements, dye incorporation by energized mitochondria was slightly greater than that of deenergized mitochondria. However, this difference in NAO uptake (15% at the most) cannot explain inhibition differences observed between state 3 and uncoupled respirations.

4. DISCUSSION

In previous reports, NAO was shown to be specific for mitochondria of HeLa cells [1] and splenocytes [2]. It was also demonstrated that the dye was accumulated in mitochondrial inner membrane independently of transmembrane potential [3]. Consequently, NAO incorporation reveals total mitochondria inner membrane surface and becomes an appropriate method for probing mitochondrial membrane in several biological fields [4,5]. However, this dye, at high concentrations, could disturb mitochondrial membrane structure and function [1,2]. For this reason, we report results concerning NAO cytotoxicity and particularly its effect on mitochondrial energetic function. At low NAO concentrations (0.1–3 μM), the inhibition of oxidative phosphorylation, determined polarographically, was recorded as a rapid loss of state 3 respirattion (fig.2). At high concentrations (3–10 μM), the phenomenon was amplified with a slight decrease in state 4 oxygen consumption. Addition of an uncoupler induced only a slight release of NAO (15% at the most) and cannot explain the different dose response curves (fig.2). As in the case of inhibition of ADP-stimulated respiration by a large number of lipophilic organic cations [16], oxygen consumption with glutamate was more sensitive to NAO than that with succinate (fig.3). This may reside in differences in the lipophilicity of the two entry sites in the respiratory chain in that the succinate site is much less lipophilic than the glutamate site [16]. Moreover the inhibition of state 3 respiration by NAO occurred without altering state 4 rate while F_0-F_1 ATPase activity was slightly prevented. The fact that NAO inhibited ADP-stimulated respiration suggests that several specific sites of inhibition are possible. Since state 4 respiration was not (or slightly) affected by NAO and state 3 respiration was inhibited with each substrate (glutamate or succinate), we considered that substrate dehydrogenases and transport were not inhibited. However, NAO strongly decreased ATP synthesis by a mechanism which is independent of P_i-transport activity but, in part, generated by the modification of adenine nucleotide translocase activity.

These findings suggest several hypotheses to explain the mechanism by which NAO affected oxidative phosphorylation.

At low concentrations, as in the case of several positively charged dyes [17–19], inhibitory effect on ADP-stimulated state 3 respiration would be a consequence of inhibition of energy-transducing activities. Inhibition by NAO has been proposed to be due to the transfer of the dye or some other cations across the mitochondrial membrane leading to the collapse of $\Delta\mu H^+$ which is the direct driving force ATP synthesis [20–24].

At high concentrations, NAO not only affected state 3 respiration and ATP synthesis but also some other reactions of oxidative phosphorylation (respiratory chain and mitochondrial carriers). Moreover, the dye acts as a noncompetitive inhibitor of ADP-stimulated respiration (result not shown). Thus, the mechanism of NAO inhibition may include some component of inner mitochondrial membrane required for optimal activity of the different enzymatic complexes. Furthermore, it has been demonstrated the absolute requirement of cardiolipin for several enzymes of inner mitochondrial membrane, particularly those involved in the ATP production process [8,25–27]. Taking into account the fact that NAO has been recently shown to specifically interact with cardiolipin [28], the inhibition of enzymatic complexes within he inner membrane would be due to the modification of the enzyme lipid environment. On the other hand this interaction of NAO with cardiolipin

might affect, like other inhibitors of ATP synthesis [29], membrane fluidity and consequently lipid-protein interactions.

We conclude that NAO inhibits oxidative phosphorylation in intact rat liver mitochondria, possibly via both interactions with cardiolipin and inhibition of energy conservation. Even though it is not possible to compare these results on mitochondria directly to those on intact cells or other subcellular fractions, these data should be of value to determine appropriate conditions for NAO use as a new vital probe.

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